Molecular Biology of the Cell
Vol. 20, 3353–3362, July 15, 2009

Induction of Dlk1 by PTTG1 Inhibits Adipocyte Differentiation and Correlates with Malignant Transformation

Águeda G. Espina,*† Cristina Méndez-Vidal,*† Miguel A. Moreno-Mateos,*† Carmen Sáez,‡ Ana Romero-Franco,* Miguel A. Japón,‡ and José A. Pintor-Toro*

*Centro Andaluz de Biología Molecular y Medicina Regenerativa, CABIMER-CSIC, 41092 Sevilla, Spain; and ‡Departamento de Anatomía Patológica, Hospital Universitario Virgen del Rocío, 41013 Sevilla, Spain

Submitted September 23, 2008; Revised April 16, 2009; Accepted May 18, 2009
Monitoring Editor: John L. Cleveland

Pituitary tumor-transforming gene-1 (PTTG1) is an oncogene highly expressed in a variety of endocrine, as well as nonendocrine-related cancers. Several tumorigenic mechanisms for PTTG1 have been proposed, one of the best characterized being its capacity to act as a transcriptional activator. To identify novel downstream target genes, we have established cell lines with inducible expression of PTTG1 and a differential display approach to analyze gene expression changes after PTTG1 induction. We identified *dlk1* (also known as *pref-1*) as one of the most abundantly expressed PTTG1 targets. Dlk1 is known to participate in several differentiation processes, including adipogenesis, adrenal gland development, and wound healing. Dlk1 is also highly expressed in neuroendocrine tumors. Here, we show that PTTG1 overexpression inhibits adipogenesis in 3T3-L1 cells and that this effect is accomplished by promoting the stability and accumulation of Dlk1 mRNA, supporting a role for PTTG1 in posttranscriptional regulation. Moreover, both *pttg1* and *dlk1* genes show concomitant expression in fetal liver and placenta, as well as in pituitary adenomas, breast adenocarcinomas, and neuroblastomas, suggesting that PTTG1 and DLK1 are involved in cell differentiation and transformation.

INTRODUCTION

Pituitary tumor transforming gene (PTTG1) encodes a multifunctional protein with roles in the control of mitosis, cell transformation, DNA repair, and gene regulation (Pei and Melmed, 1997; Zou *et al.*, 1999; Pei, 2001; Romero *et al.*, 2001; Kim *et al.*, 2007). The expression of PTTG1 in most normal tissues is very restricted, with highest levels found in the testis and thymus (Dominguez *et al.*, 1998). PTTG1 is expressed at high levels in multiple tumors including carcinomas of the lung, breast, thyroid, esophagus, colon, leukemia, and lymphoma as well as in pituitary adenomas (Saez *et al.*, 1999; Heaney *et al.*, 2000; Shibata *et al.*, 2002; Saez *et al.*, 2006). Different nonexcluding mechanisms might participate in PTTG1 tumorigenic function. PTTG1 inhibits sister-chromatid separation in vertebrates and may thereby mediate chromosome missegregation (Zou *et al.*, 1999) leading to an increase in proto-oncogene dose or to loss of heterozygosity of tumor suppressors (Jallepalli and Lengauer, 2001; Jallepalli *et al.*, 2001). Also, PTTG1 binds to Ku, the regulatory subunit of the DNA-dependent protein kinase (DNA-PK). This association is disrupted and prevented by double-strand breaks suggesting that PTTG1 connects the DNA damage-response pathway with sister chromatid separation (Romero *et al.*, 2001; Bernal *et al.*, 2008).

Furthermore, PTTG1 interacts with and inhibits p53's transcriptional activity, indicating that the oncogenic effect of PTTG1 could result from modulation of p53 functions (Bernal *et al.*, 2002). Finally, it has been proposed that PTTG1 acts as a transcriptional activator. Recently, chromatin immunoprecipitation (ChIP)-on-ChIP technology has been used to survey PTTG1-mediated regulation of 20,000 genes. These data support a role for PTTG1 in the transcriptional regulation of genes involved in a variety of cellular processes such as cell cycle, metabolic control, and signal transduction (Tong *et al.*, 2007). To examine the effect of PTTG1 on endogenous target genes, we have developed a cell line expressing PTTG1 under an inducible promoter. Among the candidate PTTG1-regulated targets analyzed, the *dlk1* gene showed the most dramatic induction. DLK1, a transmembrane protein encoded by the *dlk1* gene, belongs to a family of epidermal growth factor (EGF)-like repeat-containing proteins that include Notch/Delta/Serrate, which are involved in cell fate determination (Nichols *et al.*, 2007). DLK1 is highly expressed in preadipocytes, but its expression is abruptly down-regulated during differentiation into adipocytes (Smas and Sul, 1993; Smas *et al.*, 1998). Moreover, constitutive expression of DLK1 in 3T3-L1 cells inhibits adipocyte differentiation, whereas forced down-regulation of DLK1 by antisense expression enhances adipogenesis (Smas and Sul, 1993; Smas *et al.*, 1999). These observations point to the role of DLK1 as a growth factor, maintaining proliferating cells in an undifferentiated state. In addition, DLK1 is highly expressed in adrenal medullary neuroendocrine tumors, in neuroblastomas, small-cell lung carcinomas and

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08–09–0965) on May 28, 2009.

† These authors contributed equally to this work.

Address correspondence to: José A. Pintor-Toro (jose.pintor@cabimer.es).

Abbreviations used: *csd*, stearoyl-CoA desaturase; IPTG, isopropylthiogalactoside; *pttg1*, pituitary tumour-transforming gene.

gliomas (Laborda *et al.*, 1993; van Limpt *et al.*, 2000; Yin *et al.*, 2006), indicating that it may play a role in tumorigenesis.

In this study, we find that forced expression of PTTG1 triggers the expression of the *dlk1* gene and affects adipocyte differentiation through the regulation of DLK1 levels. Furthermore, we analyzed the levels of PTTG1 and DLK1 in some normal tissues as well as in pituitary adenomas, breast adenocarcinomas, and neuroblastomas and found a parallel expression of both proteins in all samples analyzed, indicating a potential correlation between expression of PTTG1 and DLK1 and tumor development.

MATERIALS AND METHODS

Generation of Cell Lines with Inducible PTTG1 Expression

Inducible PTTG1 (NIH3T3-PTTG1) or chloramphenicol acetyl transferase (NIH3T3-CAT) cells were generated using the LacSwitch II-inducible mammalian expression system in accordance to manufacturer's instructions (Stratagene, San Diego, CA; Wyborski and Short, 1991). Briefly, founder cell lines were generated by transfecting NIH3T3 with 10 μg of pCMVLacI, a plasmid encoding a Lac repressor protein and resistance for hygromycin. Individual hygromycin-resistant colonies were isolated and characterized by Western blot. Independent clones with the highest expression of Lac repressor were used to establish cells with inducible PTTG1 expression. Full-length human Pttg1 cDNA (Dominguez *et al.*, 1998) was cloned into the vector pOPRSVI/MCS under the control of a promoter containing both CMV and Lac operator sequences. Thus, presence of isopropylthiogalactoside (IPTG) in the culture medium induces PTTG1 expression. Selected founder cells were transfected with 10 μg of PTTG1 construct. G418-resistant cells were isolated and screened for PTTG1 expression by immunoblotting upon IPTG treatment. Three clones demonstrating tightly regulated induction of the transfected gene were selected for further studies and named NIH3T3-PTTG1. Likewise, selected founder cells were transfected with the construct pOPI3-CAT, which contained the CAT gene under CMV and Lac operator promoter sequences; these clones were named NIH3T3 control.

Differential Display

NIH3T3-PTTG1 and NIH3T3 control cells were collected at 0 and 24 h after gene induction with 2 mM of IPTG. RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 200 ng total RNA, using MMLV reverse transcriptase and one of the three anchored primers (GeneHunter, Brookline, MA). The cDNA generated was used in conjunction with the three downstream anchored primers and 32 upstream arbitrary primers (GeneHunter) for PCR display in the presence of α-[^{33}P]dATP (Fischer *et al.*, 1996). The amplified cDNAs were separated on a 6% polyacrylamide gel with urea and detected by autoradiography with x-ray film exposed overnight. Specific bands were recovered and reamplified by PCR with the same primer set and cloned into pGEM-T vector (Promega, Madison, WI). Recombinant plasmids DNAs were sequenced with Big Dye terminator kits (Applied Biosystems, Branchburg, NJ).

Northern Blot Analysis

Putative target cDNAs probes were obtained from The Resource Center German Human Genome Project (RZPD, Berlin, Germany). RNA extraction from primary tumors and hybridization conditions were described previously (Saez *et al.*, 1999). Blots were stripped and reused for the different mRNAs analyzed.

Semiquantitative and Quantitative PCR Analysis

3T3-L1 cells were seeded in six-well plates and transfected with empty vector or pcDNA3.1-Pttg1 or Foxa-2 plasmids using Lipofectamine 2000 (Invitrogen). Total RNA was prepared using TRIZOL reagent (Invitrogen). RT-PCR experiments were carried out using 1 μl of cDNAs generated from 1 μg of total RNA using Qiagen OneStep RT-PCR kit (Qiagen, Chatsworth, CA). The RT-PCR exponential phase was determined on 25–35 cycles to allow semiquantitative comparisons of cDNAs developed from identical reactions. The following primers were used: Dlk1: forward, 5′ GGAATTCTCCTGCGCGTCCTCCTGCTC3′, and reverse, 5′ CCTCGAGTTACTGTCCCTCGGTGAGGAGAG 3′; Foxa-2: forward, 5′ GTTAAAGTATGCTGGGAGCCG 3′, and reverse, 5′ CGCCCACATAGGATGACATG 3′; Pttg1: forward, 5′ AGGCACCCGTGTGGTTGCT 3′, and reverse, 5′ TAAGGCTGGTGGGGCATC 3′, and actin: forward, 5′ TTGCAATGAGCGGTTCCGCT 3′, and reverse 5′ TACAGCTGTTTGCGGATGTCC 3′. Expression of mouse and human Pttg1 mRNAs in NIH3T3 cells was determined using the Applied Biosystems 7500 Real-Time PCR System. The expression levels of the mouse mRNA Pttg1 were measured using the Applied Biosystems TaqMan Gene Expression Assays (Mm00479224_m1) and the ABI PRISM 7500 instrument (Applied Biosystems). To determine human Pttg1 mRNA levels we used two Pttg1 sequence-specific PCR primers (5′AGGCACCCGTGTGGTTGCT 3′ and 5′TAAGGCTGGTGGGGCATC 3′) and a TaqMan assay-FAM probe (5′AACTTGAGATCTCCCATCTAAGGC 3′) obtained from Operon Biotechnologies (Ebersberg, Germany). Reactions were 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. An Hprt TaqMan Gene expression assay (Mm 01545399_m1) was used to normalize variations in cDNA quantities from different samples. Measurements were carried out in quadruplicate. Absolute quantification of mRNA expression was calculated after standard curves for both human and mouse PTTG1 plasmids.

In Vitro Stability Assays

Proteins extraction and mRNA degradation assays were performed as described elsewhere with some modifications (Chakkalakal *et al.*, 2008). Briefly, NIH3T3-PTTG1 and NIH3T3 control cells were induced with 2 mM of IPTG for 20 h. Proteins extracts were prepared using 500 μl of homogenization buffer (0.01 M Tris, pH 8, 0.01 M KCl, 0.0015 M MgCl, and 2.5% IGEPAL CA-630) containing protease inhibitor complete cocktail (Roche Diagnostics, Mannheim, Germany). After homogenization, protein extracts were centrifuged at 3500 $\times$ *g* for 10 min. Then, the pellets were vortexed and incubated for 20 min on ice in 100 μl extraction buffer (0.02 Tris, pH 8, 0.45 M NaCl, 0.01 EDTA, and protease inhibitor complete cocktail). After incubation, the protein extracts were centrifuged at 14,000 $\times$ *g* for 10 min, and supernatants (enriched in nuclear and cytoskeletal fractions) were used for in vitro stability assays. Protein extracts were depleted from PTTG1 protein by incubation with 2 μg polyclonal anti-PTTG1 antibody overnight, followed by 2-h incubation with 15 μl protein A-Sepharose beads. Degradation assay were performed using total RNA (0.2 μg/μl) from NIH3T3 cells, and equal amounts (0.25 μg/μl) of NIH3T3-PTTG1 or NIH3T3 protein extracts were incubated together in degradation buffer (10 mM Tris, pH 7.4, 10 mM KOAc, 2 mM MgOAc, 2 mM DTT, 0,1 mM spermine, 1 mM ATP, 0.4 mM GTP, 10 mM phosphocreatine, 1 μg creatine phosphokinase, 40 U RNasin) for 5 or 20 min at 37°C. The reactions were stopped by addition of phenol/cloroform, and RNA was precipitated with isopropanol in the presence of glycogen (Fermentas, Vilnius, Lithuania) as a carrier. The values of Dlk1 and Hprt (as control gene) transcripts remaining at each time point were determined by quantitative real-time-PCR assay.

Lentiviral Production and Infection

The HIV packaging (pCMVDR8.91) and VSV-G (pMDG) plasmids and the lentiviral vector pHRSIN were kindly provided by Mary K. Collins (Windeyer Institute, London). The coding sequence of human PTTG1 cDNA was cloned into the vector SIN-BX-IRE/EMW, and the fragment PTTG1-IRES-enhanced green fluorescent protein (EGFP) was liberated after digestion with BamHI and NotI and subcloned into the pHRSIN vector. Insertion and orientation of the genes were confirmed by restriction enzyme digestion analyses. For production of lentivirus, 3 $\times$ 10^6 293T cells were seeded onto a 10-cm Petri dish and transfected after 24 h with Lipofectamine 2000 (Invitrogen) using 13.5 μg of transfer vector pHRSIN carrying PTTG1 cDNA, 9 μg of pCMVDR8.91, and 4.5 μg of pMDG. Lentivirus were harvested 48 h after transfection, passed through a 0.45-μm filter, and concentrated by ultracentrifugation at 100,000 $\times$ *g* for 90 min. Virus particles were resuspended in serum-free DMEM-F12 (Invitrogen), snap-frozen in liquid nitrogen, and stored at −80°C. For virus titration 2 $\times$ 10^5 293T cells were infected with virus in complete growth medium containing 8 μg/ml polybrene for 6 h. Infected cells were detected by EGFP expression using a FACScan and CellQuest software (BD Biosciences FACS Systems, San Jose, CA). For infection with the lentiviral stock, 3T3-L1 cells were plated in 60-mm plates, incubated 2 h in complete medium, and infected with lentiviral particles (MOI = 5) and 8 μg/μl polybrene for 6 h at 37°C. Media were changed to growth media without polybrene for the rest of the experiment.

Inhibition of Dlk1 Expression by siRNA

The Dlk1 mRNA sequence was analyzed for selecting inhibitory RNA sequences using standard design rules. The sequence of the selected short interfering RNA (siRNA) directed against the mouse Dlk1 mRNA was as follows: sense, 5′ GAAAGGACUGCCAGCACAAdTdT3′; and antisense, 5′ UUGUGCUGGCAGUCCUUUCdTdT 3′. An unrelated siRNA sequence was used as control (sense, 5′ UUCUCCGAACGUGUCACGUdTdT 3′; antisense, 5′ ACGUGACACGUUCGGAGAAdTdT). Both oligos were purchased from Proligo (Boulder, CO). 3T3-L1 cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer's instruction.

Western Blot Analysis

Cell lysates were prepared by lysing cells in NP40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, and NP40 at 1%, vol/vol) containing a complete cocktail of protease inhibitors (Roche Diagnostics) and 1 mM PMSF and resolved on SDS-PAGE. Immunoblotting was performed on nitrocellulose membrane according to the manufacturer's instructions. PTTG1 was detected with anti-human PTTG1 antiserum as previously described (Dominguez *et al.*, 1998). Commercially available antibodies to DLK1 (Santa Cruz Biotechnology, Santa Cruz, CA) and ß-actin (Sigma-Aldrich, St. Louis, MO) were used as recommended by the manufacturers. Signals were detected

using Enhanced Chemiluminescence Reagent ECL kit (GE Healthcare, Waukesha, WI).

Cell Culture, Flow Cytometry, and Differentiation Assays

HCT116 cells were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin-streptomycin, glutamine, and the appropriate selective agent when needed. NIH3T3 cells were grown under identical conditions except that the medium was supplemented with 10% newborn calf serum. The results were quantified from three independent experiments with three PTTG1-overexpressing NIH3T3 clones and three vector control clones. For flow cytometry analysis, at the indicated times, floating and adherent cells were washed in phosphate-buffered saline (PBS), fixed in 70% ethanol, and stained with propidium iodide (50 μg/ml). Cells were analyzed by using a FACScalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Differentiation of 3T3-L1 cells was induced by growing cells to confluence (day −2) and then shifting them to differentiation medium (DM) after 48 h (day 0). DM contained 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 5 μg/ml porcine insulin. DM lacking dexamethasone and IBMX was used as a control. Medium was changed to growth medium containing only insulin (GM, 5 μg/ml) after 48 h (day 2). Staining with Oil Red O was typically performed on day 7.

Immunohistochemistry

Tissue samples were collected from this Department of Pathology's archives and were used following the policies of the local Ethical Committee. Tumors consisted of 21 pituitary adenomas, 20 neuroblastomas, and 23 adenocarcinomas of the breast. Placental and embryonic liver tissues were obtained from spontaneous abortions at 6 and 15 wk of gestation. All tissues were Formalin-fixed and paraffin-embedded. Five-micrometer paraffin tissue sections were dewaxed, rehydrated, immersed in 3% hydrogen peroxide for 30 min, and covered with 10% normal swine serum for 15 min. Heat-induced antigen retrieval was performed using a pressure cooker and Tris-EDTA buffer, pH 9. Incubation with goat polyclonal anti-human DLK1 (1:300 dilution, Santa Cruz Biotechnology) or rabbit polyclonal anti-human PTTG1 (1:500 dilution; Dominguez *et al.*, 1998) was performed overnight at 4°C. Secondary and visualization reagents were applied using LSAB-HRP System and DAB+ (Dako, Glostrup, Denmark) according to manufacturer's recommendations. The slides were counterstained with hematoxylin and mounted in DPX (BDH

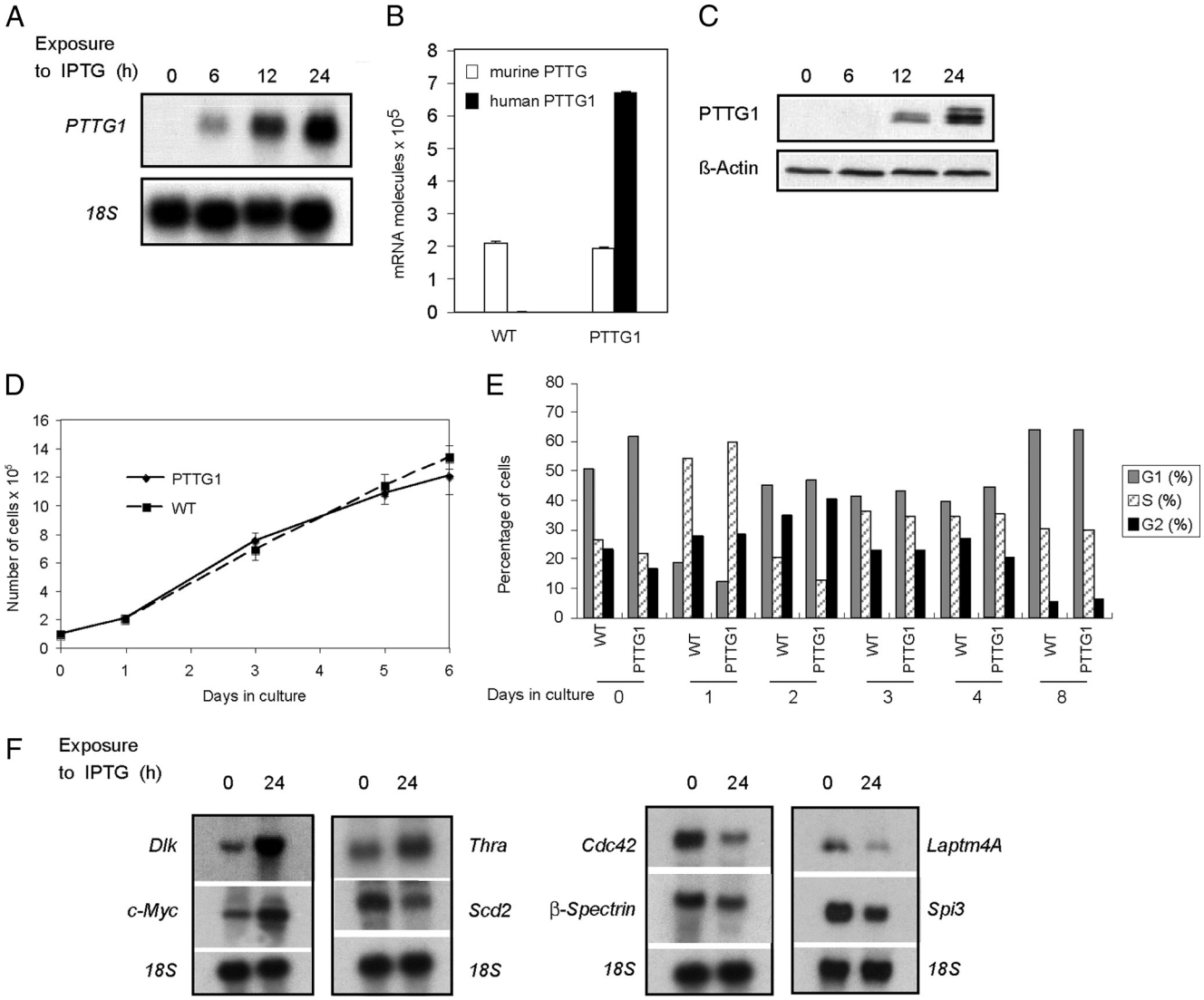

Figure 1. Inducible expression of PTTG1 and analysis of candidate target genes. (A) Northern blot analysis of Pttg1 induction after exposure to IPTG. (B) Quantification of murine and human Pttg1 mRNA in control NIH3T3 (WT) and PTTG1-overexpressing NIH3T3 cells (PTTG1), after 24 h of IPTG treatment. (C) Western blot analysis of PTTG1 induction after exposure to IPTG. (D) Proliferation rate of NIH3T3 control (WT) and PTTG1-overexpressing NIH3T3 cells (PTTG1) at various time points. Values were calculated as the average of three independent experiments using three clones of each cell line. Error bars, SE. (E) Percentage of NIH3T3 WT and PTTG1 cells in the different cell-cycle phases and at various time intervals after incubation with IPTG. (F) Representative northern blots of candidate PTTG1 targets. Total RNA obtained from PTTG1-overexpressing NIH3T3 cells grown in the presence or absence of IPTG for 24 h was analyzed using probes corresponding to some of the candidate PTTG1 targets.

Table 1. Summary of target genes expressed in PTTG1-overexpressing NIH3T3 cells

Up-regulated genes	Down-regulated genes
NM_010052: Delta-like 1 homolog (*Dlk1*)	NM_009011: RAD23UV excision repair protein homolog B (*Rad23B*)
NM_178060: Thyroid hormone receptor alpha1 (*Thra*)	NM_009019: Recombination activating protein (*Rag1*)
X03919: N-myc protooncogene	NM_011951: Mitogen-activated protein kinase p38
NM_008562: Myeloid cell leukaemia sequence 1 (*Mcl1*)	NM_009861: CDC42 GTP-binding protein (*Cdc42*)
	NM_009260: β spectrin (*Spnb2*)
	NM_009254: Serine proteinase inhibitor 3 (*Spi3*)
	NM_009768: Basigin precursor (*Bsg*)
	NM_009127: Stearoyl-coenzyme A desaturase 1 (*Scd1*)
	NM_009128: Stearoyl-coenzyme A desaturase 2 (*Scd2*)
	AK 147326: GAP-associated protein (p190)
	NM_008640: Lysosomal-associated protein transmembrane 4A (*Laptm4A*)

Laboratories, Poole, United Kingdom). Sections where primary antibody was omitted were used as negative controls. NIH3T3 cells infected with pHRSIN or pHRSIN-PTTG1 lentivirus were grown on coverslips, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.1% Triton X-100. Immunostaining was carried out according to standard protocols. The goat polyclonal anti-human DLK1 and rabbit polyclonal anti-human PTTG1, mentioned above, were used as primary antibodies. Alexa (blue) 350–conjugated donkey anti-goat IgG and Alexa (green) 488–conjugated donkey anti-rabbit IgG (Molecular Probes, Invitrogen, Eugene, OR) were used as secondary antibodies.

RESULTS

Inducible Expression of Pttg1 and Analysis of Candidate Target Genes

To identify PTTG1 target genes, we established NIH3T3 cells expressing PTTG1 under an IPTG-responsive promoter (Wyborski and Short, 1991). Northern and Western blot analyses showed tightly regulated induction of Pttg1. Pttg1 mRNA was undetectable in cells growing in the absence of IPTG, but treatment with 2 mM IPTG led to the induction of the expected 0.7 kb Pttg1 transcript (Figure 1A). Quantitative analysis revealed that total Pttg1 transcripts were about fourfold higher in IPTG-treated cells (PTTG1) than in untreated cells (WT; Figure 1B). Analysis of protein expression using specific anti-PTTG1 antibodies also showed induction of the expected full-length PTTG1 protein, after treatment with IPTG (Figure 1C). Protein levels were determined using specific antibodies and both purified mouse and human PTTG1 proteins. Untreated NIH3T3 cells contained 14 pg of PTTG1 per μg of total cellular protein and this concentration

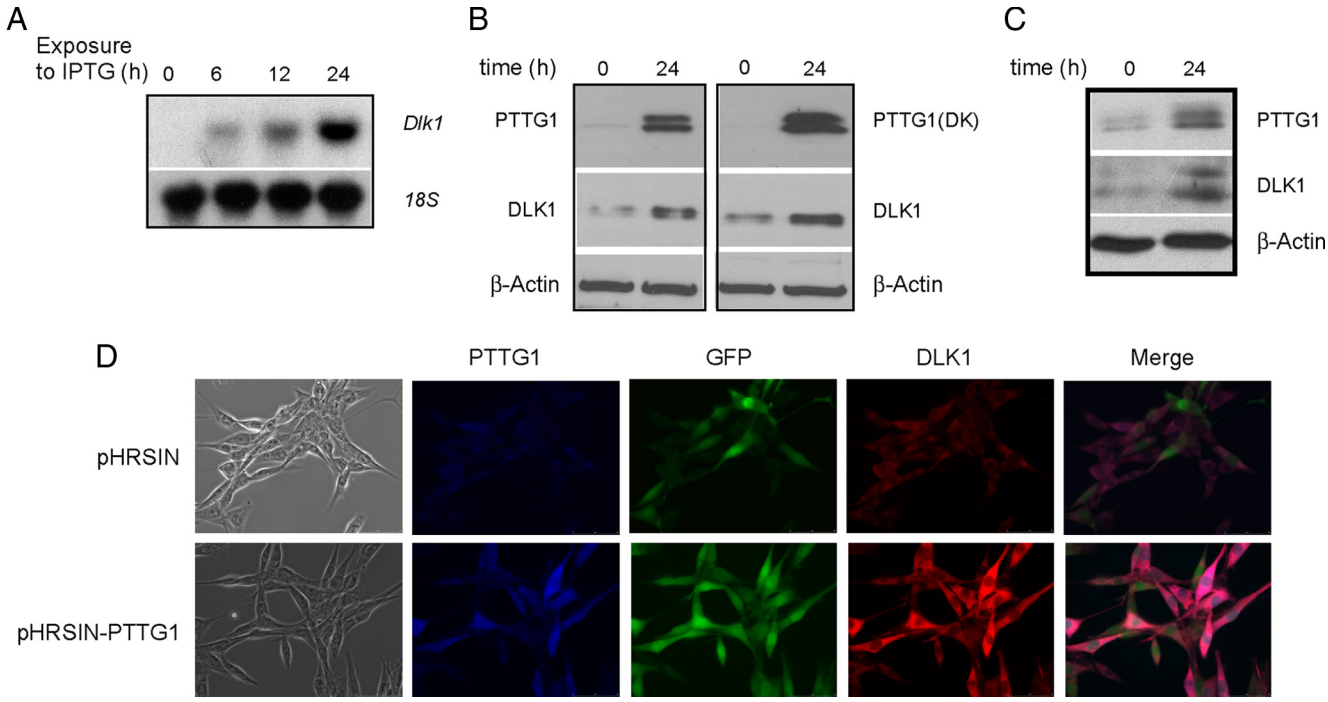

Figure 2. Induction of Dlk1 by PTTG1. (A) Northern blot analysis of Dlk1 induction in NIH3T3 cells after IPTG-induced PTTG1 expression. (B) Western blot analysis of DLK1 expression in both PTTG1- and PTTG1 (DK)-NIH3T3 cells after 24 h of treatment with IPTG. Samples showed in these panels were resolved on the same gel, and the results obtained were in the same immunoblot. (C) Western blot analysis of DLK1 levels in HCT116 cells infected with a lentivirus vector expressing PTTG1 cDNA under a constitutive promoter. (D) Immunocytochemical detection of PTTG1 and DLK1 in NIH3T3 cells infected either with lentivirus containing GFP cDNA (pHRSIN) or lentivirus containing the cassette GFP-IRES-PTTG1 (pHRSIN-PTTG1) under a constitutive promoter. Cells were triple stained for PTTG1 (blue), GFP (green), and DLK1 (red).

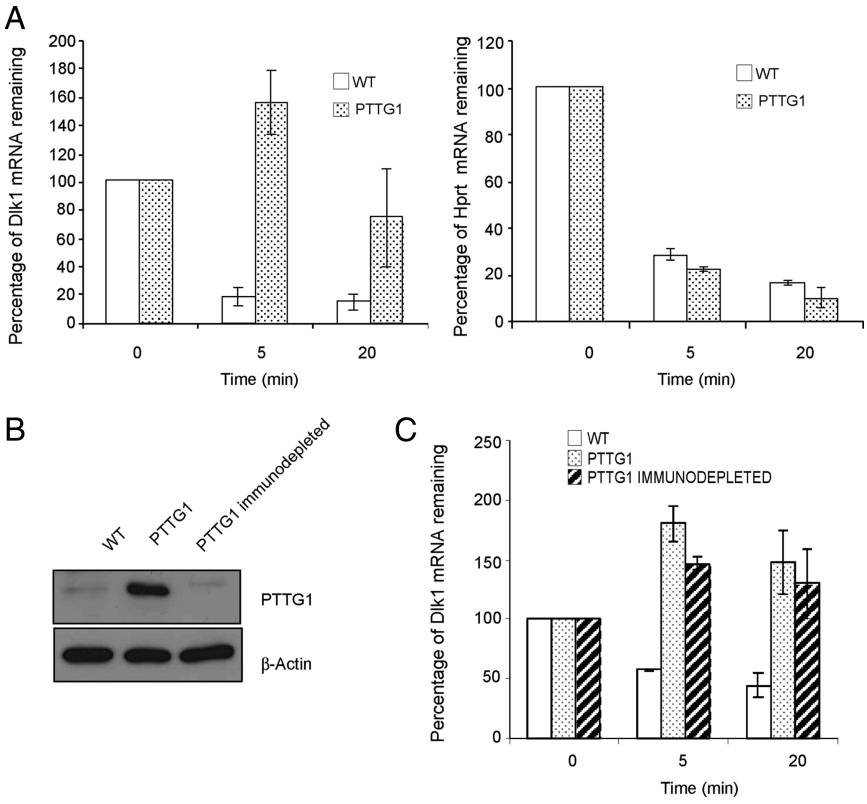

Figure 3. PTTG1 overexpression stabilizes Dlk1 mRNA. (A) Quantification of Dlk1 and Hprt mRNA levels after incubation of total RNA with WT-NIH3T3 (WT) or PTTG1-NIH3T3 (PTTG1) protein extracts. Error bars, SE from three independent experiments. (B) Western blot analysis of PTTG1 protein levels in WT-NIH3T3 (WT) and PTTG1-NIH3T3 (PTTG1) extracts and PTTG1-NIH3T3 extracts preincubated with anti-PTTG1 antibody and protein A-Sepharose beads (PTTG1 immunodepleted). (C) Quantification of Dlk1 mRNA levels after incubation of total RNA with WT-NIH3T3, PTTG1-NIH3T3, and PTTG1 immunodepleted PTTG1-NIH3T3 extracts. Error bars, SE from three independent experiments.

was 9.5-fold higher after 18 h of treatment with IPTG (data not shown). To determine the effect of PTTG1 induction in NIH3T3 cells, we measured the level of cell proliferation, as well as the percentage of cells in the different phases of the cell cycle. We did not observe any significant differences between induced and noninduced cells (Figure 1, D and E).

The carboxy-terminal domain of PTTG1 is able to display a strong transcriptional activity on transiently transfected reporter constructs in yeast and mammalian cells (Dominguez *et al.*, 1998), suggesting a role in transcriptional regulation. We used a differential display approach (Liang and Pardee, 1992) to search for endogenous genes whose expression could be altered after inducible expression of PTTG1. cDNAs corresponding to 69 different genes were identified. Northern blot analysis confirmed that 15 of these genes were differentially expressed, and most of them had not previously been identified as PTTG1 targets (Table 1 and Figure 1F).

Induction of dlk1 by PTTG1

Among the differentially expressed genes, we identified *dlk1* as the most dramatically induced target after PTTG1 induction. Time-course expression of Dlk1 mRNA after PTTG1 induction in NIH3T3 cells was coincident with that of Pttg1, being detectable 6 h after IPTG treatment (Figure 2A). Furthermore, immunoblotting analysis using cell lysates from the inducible cell line also showed induction of DLK1 protein (Figure 2B). An increase in DLK protein levels was also observed in cells overexpressing a stable form of PTTG1, PTTG1 (DK), which contains mutated KEN and destruction boxes (Figure 2B). These domains are necessary for degradation of PTTG1 via the proteosome. To exclude cell type-specific effects of PTTG1 on Dlk1 expression, we used the HCT116 human cell line of epithelial origin and lentiviral-based delivery of Pttg1. In these cells, induction of DLK1 by PTTG1 was also observed (Figure 2C), indicating that the signaling pathways leading to DLK1 up-regulation are functional in both cell lines analyzed. Increased levels of DLK1 after overexpression of PTTG1 protein were also detected by immunofluorescence. Figure 2D shows accumulation of DLK1 in NIH3T3 cells infected with pHRSIN-PTTG1 lentivirus, which contain the cassette *PTTG1-IRES-GFP* under a constitutive promoter in comparison to cells infected with pHRSIN expressing the GFP gene. We next analyzed whether PTTG1 acts as a transcriptional regulator of the *dlk1* gene using reporter plasmids containing the *dlk1* promoter. Control and PTTG1-overexpressing NIH3T3 cells were transiently transfected with the luciferase reporter plasmids DLK-191, DLK-1400, and also DLK-4200, which contain the *dlk1* promoter extending 191, 1400, and 4062 base pairs upstream of the transcriptional start site, respectively. These *dlk1* promoter regions showed no significant changes in the promoter activity between PTTG1-overexpressing NIH3T3 and mock-transfected NIH3T3 (Supplemental Figure S1). We conclude that the promoter fragments used in these experiments do not contain PTTG1 response elements.

To determine whether the higher level of Dlk1 mRNA in PTTG1-overexpressing cells was due to increased Dlk1 mRNA stability, we chose to employ a previously described in vitro stability assay (Chakkalakal *et al.*, 2008). With the use of this assay, we examined the degradation of Dlk1 mRNA in the presence of protein extracts obtained from PTTG1-overexpressing cells or control cells. As shown in Figure 3A, Dlk1 mRNA degrades at a slower rate in the presence of protein extracts from PTTG1-overexpressing cells, in comparison to mRNA incubated with control cell extracts. We observed no difference in the rate of degradation of the housekeeping gene Hprt transcripts upon incubation with either control or PTTG1-overexpressing cells extracts. In

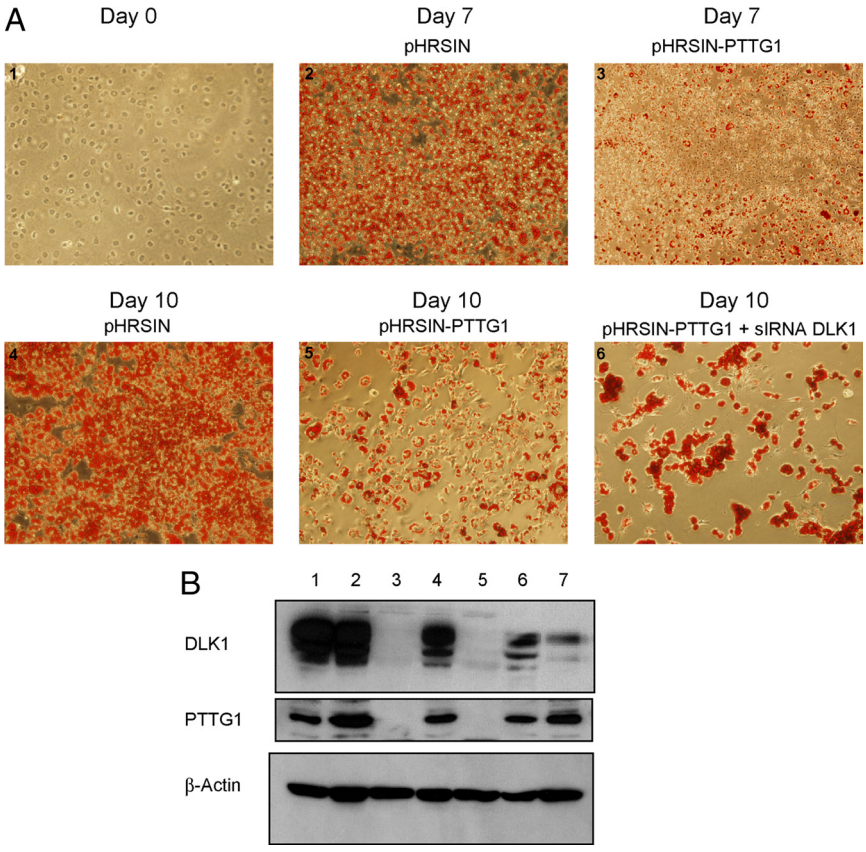

Figure 4. High PTTG1 levels inhibit adipocyte differentiation. (A) Oil Red O staining of fat droplets on day 7 (panels 2 and 3) and 10 (panels 4–6) of differentiating cells. Cells shown in panels 2 and 4 were transferred to the differentiation medium after infection with pHRSIN control lentivirus, whereas cells in panels 3, 5, and 6 were transferred to the differentiation medium after infection with pHRSIN-PTTG1 lentivirus. Cells in panel 6 were transfected with Dlk1 siRNA on day 7 and Oil Red O stained on day 10. Panel 1 corresponds to Oil Red O staining of undifferentiated cells. (B) Analysis of DLK1, PTTG1, and β-actin (loading control) levels by Western immunoblotting in mouse preadipocytes 3T3-L1 cultured under selective conditions to induce cell differentiation. Lane 1, cells on day 0; lanes 3 and 5, cells on days 7 and 10 following differentiation induction after infection with pHRSIN lentivirus; lanes 2, 4, and 6, cells on days 0, 7, and 10 after the induction of differentiation after infection with pHRSIN-PTTG1 lentivirus; lane 7, corresponds to cells on day 10 after differentiation induction infected with pHRSIN-PTTG1 lentivirus and transfected on day 7 with a specific siRNA directed against Dlk1 mRNA.

an attempt to demonstrate that PTTG1 was directly involved in the Dlk1 mRNA stabilization, we immunodepleted PTTG1 from PTTG1-overexpressing cells extracts. We used a double incubation with anti-PTTG1 antibody for the depletion. The efficiency of immunodepletion was estimated to be >95% by immunoblotting (Figure 3B). PTTG1-depleted extracts kept the capacity to stabilize the Dlk1 mRNA (Figure 3C). This result suggests that PTTG1 does not interact with mRNA Dlk1 and that one or more proteins induced by PTTG1 contribute to the differential abundance of DLK1 transcripts in PTTG1-overexpressing cells.

PTTG1 Signaling Inhibits Differentiation of 3T3-L1 Cells

DLK1 is a critical regulator of adipogenesis. To investigate the effect of PTTG1 signaling on adipocyte differentiation, we used a lentivirus expressing Pttg1 cDNA in 3T3-L1 cells. These cells, when grown to confluence and exposed to a differentiation-promoting medium (containing IBMX, insulin, and dexamethasone), undergo adipogenesis and accumulate lipids, which can be visualized after Oil Red O staining. 3T3-L1 cells infected with a control lentivirus (pHRSIN) normally followed this process (Figure 4A, panels 2 and 4), and showed down-regulation of DLK1 (Figure 4B, lanes 3 and 5). Remarkably, PTTG1 expression was concurrently abolished during differentiation of 3T3-L1 cells (Figure 4B, lanes 3 and 5). However, when 3T3-L1 cells were infected with pHRSIN-PTTG1 lentivirus, they accumulated both PTTG1 and DLK1 proteins (Figure 4B, lanes 2, 4, and 6) and significantly accumulated lower amounts of lipids (Figure 4A, panels 3 and 5). Importantly, this effect was overcome in cells transfected with a specific siRNA directed against Dlk1 (Figure 4A, panel 6, and B, lane 7) suggesting that PTTG1 blocks 3T3-L1 differentiation via DLK1 accumulation. Likewise, the expression level of the adipocyte markers *scd1* and *scd2* (stearoyl-CoA desaturase 1 and 2) decreases notably after PTTG1 overexpression (Figure 1B and Table 1). However, infection of 3T3-L1 cells with a lentivirus containing a specific siRNA against PTTG1 did not show a significant decrease of Dlk1 levels and exhibited a similar differentiation capacity compared with control GFP-expressing cells (Supplemental Figure S2). These data suggest the involvement of additional signaling pathways involved in the stabilization of DLK1 levels in these cells.

PTTG1 and FOXA-2 Regulate Dlk1 Levels through Independent Pathways

Previous studies have demonstrated that in preadipocytes, the transcription factor FOXA-2 inhibits adipocyte differentiation by activating the transcription of the *dlk1* gene (Wolfrum *et al.*, 2003). To determine the possibility that DLK1, PTTG1, and FOXA-2 take part in the same regulatory pathway, 3T3-L1 cells were transfected with plasmids expressing either Pttg1 or Foxa-2 cDNAs under the control of a constitutive promoter. Expression levels of Pttg1, Foxa-2, and Dlk1 were measured by semiquantitative RT-PCR. In agreement with our and previous results, Dlk1 expression was increased in both Pttg1- and Foxa-2–transfected cells (Figure 5). However, no differences in Pttg1 mRNA expression were found in 3T3-L1 cells overexpressing FOXA-2. Similarly, the expression levels of Foxa-2 were not affected by PTTG1 overexpression, suggesting that PTTG1 and FOXA-2 regulate Dlk1 levels through independent pathways.

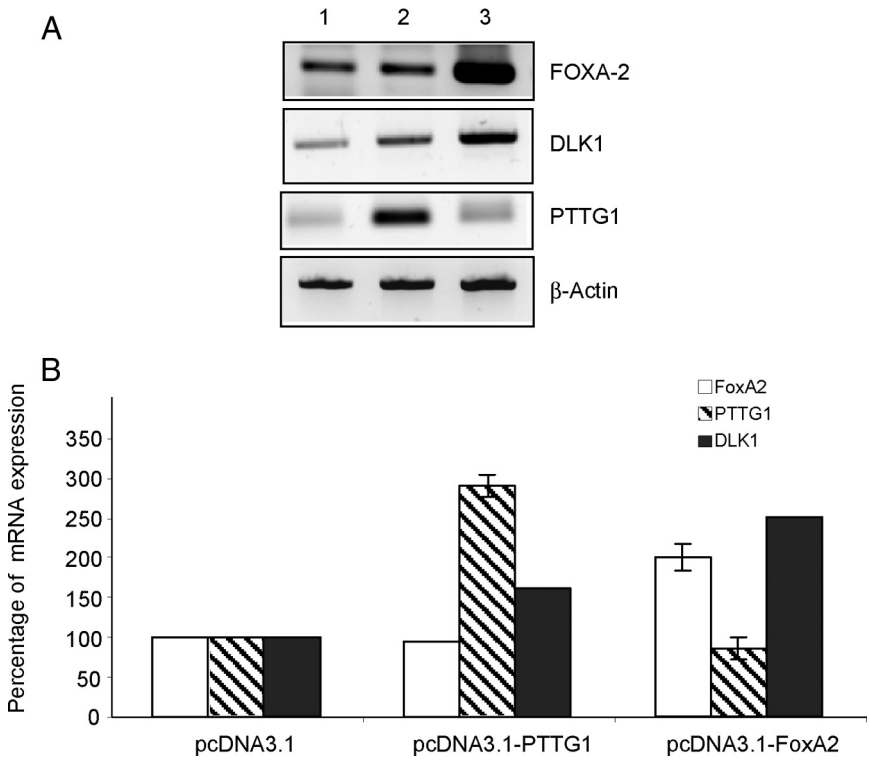

Figure 5. FOXA-2 and PTTG1 regulate Dlk1 levels via independent pathways. (A) Semiquantitative RT-PCR analysis of Foxa-2, Pttg1, and Dlk1 expression after ectopic pcDNA3.1 vector (lane 1), Pttg1 (lane 2), and Foxa-2 (lane 3) expression in 3T3-L1 cells. β-Actin was used as internal control. A representative result of three independent experiments is shown. (B) Densitometric analysis of electrophoretic bands. Intensities were normalized to the β-actin signal. Errors bars, SE of three replicate experiments.

Concurrent Expression of PTTG1 and DLK1 in Normal and Tumor Tissues

Dlk1 mRNA expression was tested by Northern blot in a series of normal human tissues among which placenta exhibited maximal levels (Figure 6A). Immunohistochemical studies showed that 6-wk placental trophoblasts and embryonic liver present similar levels of PTTG1 and DLK1 proteins (Figure 6, D–G). Concordantly, expression of both proteins was reduced at 15 wk of gestation (Figure 6, H and I), with DLK1 expression retained in hematopoietic cells. As it was shown in Figure 4B, DLK1 and PTTG1 proteins were undetectable by Western blot in adipocytes differentiated in vitro; likewise, these proteins were also absent in perithymic differentiated adipocytes (Figure 6, B and C). In addition, expression of Pttg1 and Dlk1 mRNAs was studied by Northern blot in a set of pituitary adenomas and breast adenocarcinomas. We analyzed nine surgically removed adenomas of pituitary and three normal pituitary postmortem specimens. We detected high levels of Pttg1 mRNA in all adenomas (Figure 6J, lanes 1–9), whereas a very weak or undetectable signal was observed in normal pituitary specimens (Figure 6J, lanes 10–12). Strikingly, Dlk1 mRNA showed concomitant expression to Pttg1 mRNA in these samples (Figure 6J). In breast tissue, Pttg1 mRNA was abundantly expressed in all adenocarcinomas tested compared with normal breast tissue (Figure 6K, lanes 1–12). Similarly to the situation found in the pituitary adenomas, we also observed a correlation between the expression of Pttg1 and Dlk1 mRNA in breast adenocarcinomas. However, despite the high Pttg1 levels found in all the tumor samples, we found intermediate Dlk1 expression in two adenocarcinomas (Figure 6K, lanes 5 and 6) and a weaker signal in four samples (Figure 6K, lanes 1 and 10–12). To confirm these results, we performed immunohistochemical studies in 21 pituitary adenomas and 23 breast adenocarcinomas. In pituitary adenomas, parallel expression of PTTG1 and DLK1 was detected in 80.9% of cases (Figure 6, L and M) and 73.9% of breast adenocarcinomas (Figure 6, N and O). In neuroblastomas, immunohistochemical experiments (n = 20) also demonstrated concordant expression of PTTG1 and DLK1 in 80% cases (Figure 6, P and Q).

DISCUSSION

Different mechanisms have been proposed to explain PTTG1 tumorigenic capabilities, including its role as a transcriptional activator. To address the functional properties of PTTG1 and to examine its effects on endogenous target genes, we analyzed a set of genes whose expression is affected by high levels of PTTG1, focusing our attention on the *dlk1* gene, which showed the highest levels of induction. Numerous studies have shown that forced expression of *dlk1* inhibits adipogenesis, whereas its suppression promotes this process, pointing to an important role for *dlk1* in the maintenance of the undifferentiated state of preadipocytes. The high levels of Dlk1 found after overexpression of PTTG1 in NIH3T3 cells, prompted us to analyze the effect of PTTG1 on adipocyte differentiation. Here we show that constitutive lentiviral-mediated expression of PTTG1 maintains the expression levels of DLK1 and suppress adipocyte differentiation, suggesting a role for PTTG1 in this process. Although overexpression of PTTG1 affects the expression levels of numerous genes, we observed that ectopic expression of PTTG1, followed by a decrease of Dk1 expression using a specific siRNA, leads to progression of differentiation, indicating that the effect of PTTG1 on adipocyte differentiation is specifically mediated through the regulation of *dlk1*.

Using an in vitro stability assay, we have determined that Dlk1 mRNA degrades at a slower rate in the presence of protein extracts enriched in PTTG1 protein. We believe that this effect is not achieved through direct binding of PTTG1 to Dlk1 mRNA because PTTG1 amino acid sequence lacks

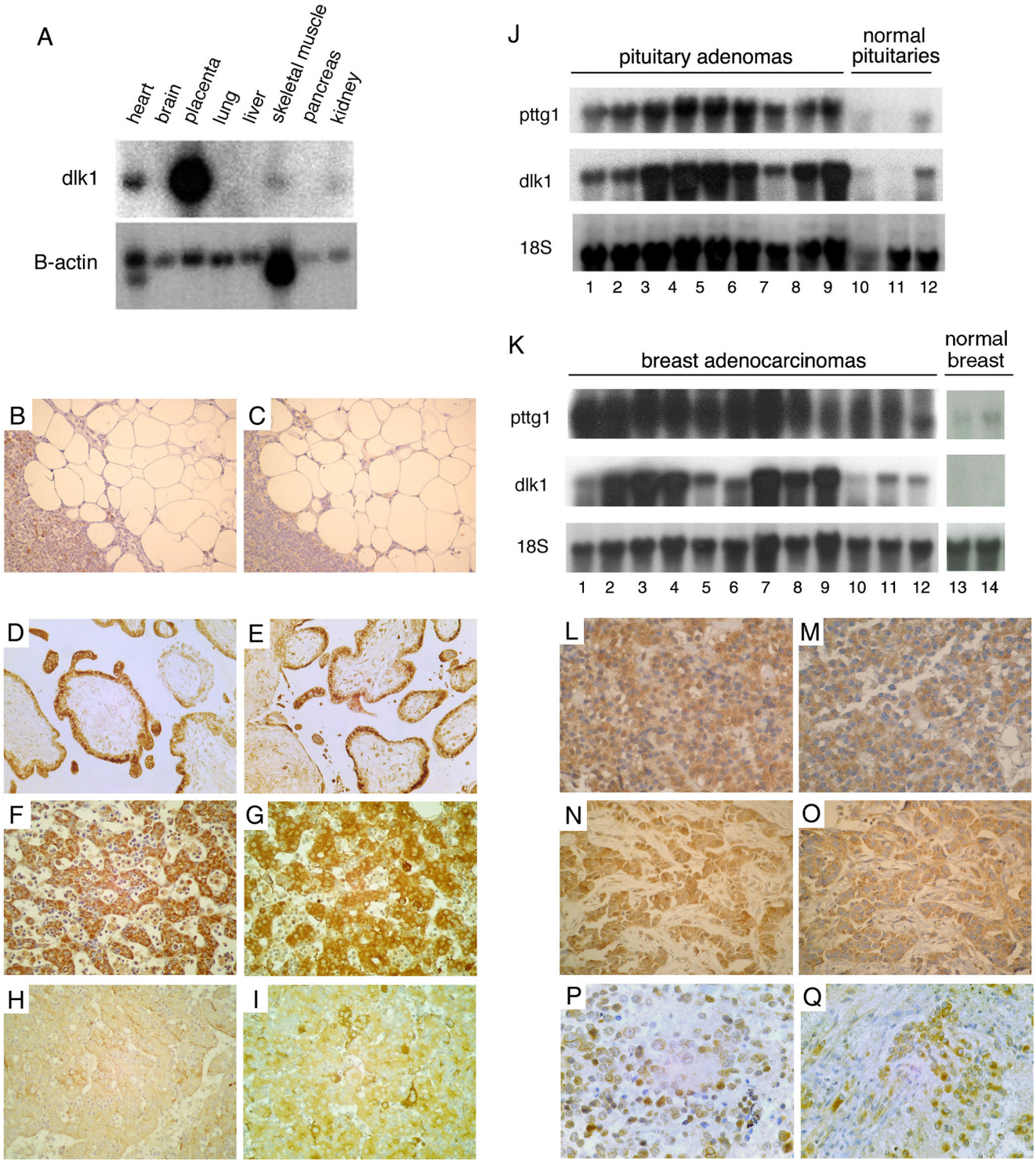

Figure 6. PTTG1 and DLK1 expression in normal and tumor tissues. (A) Northern blot analysis of Poly (A)+ mRNA derived from various adult human tissues (Clontech) hybridized with Dlk1- and β-actin–specific probes. (B and C) Immunohistochemical expression of PTTG1 (B) and DLK1 (C) in perithymic differentiated adipocytes (200×). (D–I) Immunohistochemical expression of PTTG1 (D, F, and H) and DLK1 (E, G, and I) in human placenta (200×; D and E); human liver at 6 wk (F and G) and 15 wk (H and I) of gestation (200×). (J and K) Northern blot analysis of Pttg1 and Dlk1 mRNA expression in pituitary adenomas (J) and breast adenocarcinomas (K). (L–Q) Immunohistochemical demonstration of PTTG1 (L, N, and P) and DLK1 (M, O, and Q) in pituitary adenoma (L and M; 400×), breast adenocarcinoma (N and O; 200×), and neuroblastoma (P and Q; 400×).

putative RNA-binding domains. Furthermore, the stability of Dlk1 mRNA in PTTG1-overexpressing NIH3T3 cells extracts is not affected by depletion of PTTG1 from these extracts. This suggests that Dlk1 mRNA stability is indirectly

modulated by PTTG1. In fact, expression of genes coding for RNA-binding proteins, like *rbms3*, which stabilizes specific mRNAs (Fritz and Stefanovic, 2007), is notably increased in PTTG1-overexpressing NIH3T3 cells. To test whether PTTG1 was able to transactivate the *dlk1* gene, we performed luciferase reporter assays using Dlk1 promoter fragments of 191, 1400, or 4200 base pairs. Under these experimental conditions PTTG1 does not seem to act as a transcriptional activator of *dlk1*, although we cannot rule out the presence of PTTG1 response elements in other regions of the *dlk1* gene and that both a transcriptional and posttranscriptional mechanisms could act in concert to promote increased expression of Dlk1.

A cascade of transcription factors involved in adipocyte differentiation has been identified, among them, the hepatocyte nuclear factor FOXA-2. This transcription factor is expressed in preadipocytes and inhibits the differentiation of this type of cells by activating the transcription of the *dlk1* gene (Wolfrum *et al.*, 2003). As in the case of PTTG1 overexpression, DLK1 is required for the inhibitory action of FOXA2 on adipocyte differentiation. In fact, antibodies against the bioactive soluble form of DLK1 block the differentiation of 3T3-L1 cells constitutively expressing the *foxa-2* gene (Smas *et al.*, 1997). To study the role of FOXA-2 in PTTG1-mediated DLK1 induction, we performed quantitative and semiquantitative RT-PCR analysis of Pttg1, Dlk1, and Foxa-2 mRNAs in 3T3-L1 cells. Under this experimental setting, we found that FOXA-2 does not affect the expression level of Pttg1 and vice versa suggesting that PTTG1 and FOXA-2 participate in the induction of Dlk1 through different pathways. However, both FOXA-2 and DLK1 expression can be enhanced in primary preadipocytes by the growth hormone (GH), suggesting a FOXA-2–dependent antiadipogenic activity of GH. Likewise, a positive correlation between PTTG1 expression and both in vitro and in vivo GH secretion has been demonstrated (Hunter *et al.*, 2003). Therefore, we cannot exclude the possibility that PTTG1 and FOXA-2–dependent mechanisms leading to DLK1 induction are connected via the GH.

A parallel expression of PTTG1 and DLK1 was observed at early stages of human fetal liver development and in placenta. These expression data support a role for *dlk1* and *pttg1* in maintaining proliferating cells in the undifferentiated state and indicate that these genes may also play a role in the regulation of the physiological exchange that occurs within the placenta. Most strikingly, we show that DLK1 and PTTG1 are also closely expressed in tumors. Expression of Dlk1 in human tumors has been previously reported in neuroblastoma and small cell lung carcinoma cell lines (Laborda *et al.*, 1993) and, more recently, in pituitary adenomas and pheochromocytomas (Altenberger *et al.*, 2006). DLK1 may have oncogenic properties as increased expression of DLK1 in glioblastoma multiforme cell lines enhances their transformed phenotype (Yin *et al.*, 2006). High PTTG1 levels have been also reported in a variety of endocrine- and nonendocrine-related cancers (Saez *et al.*, 1999; Heaney *et al.*, 2000; Kakar and Malik, 2006; Saez *et al.*, 2006). Furthermore, PTTG1 levels correlate with tumor invasiveness (Heaney *et al.*, 2000; Saez *et al.*, 2006), and it has been identified as a key signature gene associated with tumor metastasis (Ramaswamy *et al.*, 2003). Interestingly, we found that PTTG1 and DLK1 proteins, whose expression is limited to a small number of specific tissues in the adult, were concurrently expressed in ~80% of pituitary adenomas and neuroblastomas and 74% of breast adenocarcinomas. This indicates that, similarly to PTTG1, DLK1 gene expression could be of prognostic interest in future clinical studies involving these tumor types.

Gene expression studies evidence that embryonic programs reemerge during the development of some tumors and that aggressive tumor cells are phenotypically plastic, sharing many properties with embryonic cells (Gupta *et al.*, 2005; Topczewska *et al.*, 2006). We propose that in malignant tumors, coexpression of PTTG1 and DLK1, physiologically expressed during fetal growth and development, is associated with the regression to a more primitive, fetal-like phenotype.

To our knowledge, this is the first demonstration that overexpression of PTTG1 may affect the differentiation process of adipocytes. Importantly, we also provide evidence supporting a role for PTTG1 in post-transcriptional regulation. Furthermore, our findings highlight the convergence of tumorigenic and embryonic signaling pathways. The regained ability of PTTG1 to promote the accumulation of Dlk1, a developmentally regulated protein, strongly supports the idea that PTTG1 may increase the level of undifferentiation and, therefore, the aggressiveness of the some tumors.

ACKNOWLEDGMENTS

We thank Jorge Ferrer (Department of Endocrinology, Hospital Clínic i Universitari, Barcelona, Spain) for providing the Foxa-2 cDNA. J.A.P.-T. and M.A.J. were supported by grants from the Spanish Ministerio de Educación y Ciencia (SAF 2005-07713-C03-02 and -03) and the DGUI of the Junta de Andalucía. C.M.-V. was a recipient of a postdoctoral contract ("Juan de la Cierva" program) from the Spanish Ministerio de Educación y Ciencia. C.S. was supported by a contract from Instituto de Salud Carlos III/FIS.

REFERENCES

Altenberger, T., Bilban, M., Auer, M., Knosp, E., Wolfsberger, S., Gartner, W., Mineva, I., Zielinski, C., Wagner, L., and Luger, A. (2006). Identification of DLK1 variants in pituitary- and neuroendocrine tumors. Biochem. Biophys. Res. Commun. *340*, 995–1005.

Bernal, J. A., Luna, R., Espina, A., Lazaro, I., Ramos-Morales, F., Romero, F., Arias, C., Silva, A., Tortolero, M., and Pintor-Toro, J. A. (2002). Human securin interacts with p53 and modulates p53-mediated transcriptional activity and apoptosis. Nat. Genet. *32*, 306–311.

Bernal, J. A., Roche, M., Mendez-Vidal, C., Espina, A., Tortolero, M., and Pintor-Toro, J. A. (2008). Proliferative potential after DNA damage and nonhomologous end joining are affected by loss of securin. Cell Death Differ. *15*, 202–212.

Chakkalakal, J. V., Miura, P., Belanger, G., Michel, R. N., and Jasmin, B. J. (2008). Modulation of utrophin A mRNA stability in fast versus slow muscles via an AU-rich element and calcineurin signaling. Nucleic Acids Res. *36*, 826–838.

Dominguez, A., Ramos Morales, F., Romero, F., Rios, R. M., Dreyfus, F., Tortolero, M., and Pintor Toro, J. A. (1998). hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG. Oncogene *17*, 2187–2193.

Fischer, R., Wei, Y., and Berchtold, M. (1996). Detection of calmodulin-binding proteins using a ^{32}P-labeled GST-calmodulin fusion protein and a novel renaturation protocol. Biotechniques *21*, 292–296.

Fritz, D., and Stefanovic, B. (2007). RNA-binding protein RBMS3 is expressed in activated hepatic stellate cells and liver fibrosis and increases expression of transcription factor Prx1. J. Mol. Biol. *371*, 585–595.

Gupta, P. B., Kuperwasser, C., Brunet, J. P., Ramaswamy, S., Kuo, W. L., Gray, J. W., Naber, S. P., and Weinberg, R. A. (2005). The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. Nat. Genet. *37*, 1047–1054.

Heaney, A. P., Singson, R., McCabe, C. J., Nelson, V., Nakashima, M., and Melmed, S. (2000). Expression of pituitary-tumour transforming gene in colorectal tumours [see comments]. Lancet *355*, 716–719.

Hunter, J. A., Skelly, R. H., Aylwin, S. J., Geddes, J. F., Evanson, J., Besser, G. M., Monson, J. P., and Burrin, J. M. (2003). The relationship between pituitary tumour transforming gene (PTTG) expression and in vitro hormone

and vascular endothelial growth factor (VEGF) secretion from human pituitary adenomas. Eur. J. Endocrinol. *148*, 203–211.

Jallepalli, P. V., and Lengauer, C. (2001). Chromosome segregation and cancer: cutting through the mystery. Nat. Rev. Cancer *1*, 109–117.

Jallepalli, P. V., Waizenegger, I. C., Bunz, F., Langer, S., Speicher, M. R., Peters, J. M., Kinzler, K. W., Vogelstein, B., and Lengauer, C. (2001). Securin is required for chromosomal stability in human cells. Cell *105*, 445–457.

Kakar, S. S., and Malik, M. T. (2006). Suppression of lung cancer with siRNA targeting PTTG. Int. J. Oncol. *29*, 387–395.

Kim, D. S., *et al.* (2007). Securin induces genetic instability in colorectal cancer by inhibiting double-stranded DNA repair activity. Carcinogenesis *28*, 749–759.

Laborda, J., Sausville, E. A., Hoffman, T., and Notario, V. (1993). dlk, a putative mammalian homeotic gene differentially expressed in small cell lung carcinoma and neuroendocrine tumor cell line. J. Biol. Chem. *268*, 3817–3820.

Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967–971.

Nichols, J. T., Miyamoto, A., and Weinmaster, G. (2007). Notch signaling—constantly on the move. Traffic *8*, 959–969.

Pei, L. (2001). Identification of c-myc as a down-stream target for pituitary tumor-transforming gene. J. Biol. Chem. *276*, 8484–8491.

Pei, L., and Melmed, S. (1997). Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol. Endocrinol. *11*, 433–441.

Ramaswamy, S., Ross, K. N., Lander, E. S., and Golub, T. R. (2003). A molecular signature of metastasis in primary solid tumors. Nat. Genet. *33*, 49–54.

Romero, F., Multon, M. C., Ramos-Morales, F., Dominguez, A., Bernal, J. A., Pintor-Toro, J. A., and Tortolero, M. (2001). Human securin, hPTTG, is associated with Ku heterodimer, the regulatory subunit of the DNA-dependent protein kinase. Nucleic Acids Res. *29*, 1300–1307.

Saez, C., Japon, M. A., Ramos Morales, F., Romero, F., Segura, D. I., Tortolero, M., and Pintor Toro, J. A. (1999). hpttg is over-expressed in pituitary adenomas and other primary epithelial neoplasias. Oncogene *18*, 5473–5476.

Saez, C., Martinez-Brocca, M. A., Castilla, C., Soto, A., Navarro, E., Tortolero, M., Pintor-Toro, J. A., and Japon, M. A. (2006). Prognostic significance of human pituitary tumor-transforming gene immunohistochemical expression in differentiated thyroid cancer. J. Clin. Endocrinol. Metab. *91*, 1404–1409.

Shibata, Y., *et al.* (2002). Expression of PTTG (pituitary tumor transforming gene) in esophageal cancer. Jpn. J. Clin. Oncol. *32*, 233–237.

Smas, C. M., Chen, L., and Sul, H. S. (1997). Cleavage of membrane-associated pref-1 generates a soluble inhibitor of adipocyte differentiation. Mol. cell. biol. *17*, 977–988.

Smas, C. M., Chen, L., Zhao, L., Latasa, M. J., and Sul, H. S. (1999). Transcriptional repression of pref-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation. J. Biol. Chem. *274*, 12632–12641.

Smas, C. M., Kachinskas, D., Liu, C. M., Xie, X., Dircks, L. K., and Sul, H. S. (1998). Transcriptional control of the pref-1 gene in 3T3-L1 adipocyte differentiation. Sequence requirement for differentiation-dependent suppression. J. Biol. Chem. *273*, 31751–31758.

Smas, C. M., and Sul, H. S. (1993). Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell *73*, 725–734.

Tong, Y., Tan, Y., Zhou, C., and Melmed, S. (2007). Pituitary tumor transforming gene interacts with Sp1 to modulate G1/S cell phase transition. Oncogene.

Topczewska, J. M., Postovit, L. M., Margaryan, N. V., Sam, A., Hess, A. R., Wheaton, W. W., Nickoloff, B. J., Topczewski, J., and Hendrix, M. J. (2006). Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. Nat. Med. *12*, 925–932.

van Limpt, V., Chan, A., Caron, H., Sluis, P. V., Boon, K., Hermus, M. C., and Versteeg, R. (2000). SAGE analysis of neuroblastoma reveals a high expression of the human homologue of the *Drosophila* Delta gene. Med. Pediatr. Oncol. *35*, 554–558.

Wolfrum, C., Shih, D. Q., Kuwajima, S., Norris, A. W., Kahn, C. R., and Stoffel, M. (2003). Role of Foxa-2 in adipocyte metabolism and differentiation. J. Clin. Invest. *112*, 345–356.

Wyborski, D. L., and Short, J. M. (1991). Analysis of inducers of the *E. coli* lac repressor system in mammalian cells and whole animals. Nucleic Acids Res. *19*, 4647–4653.

Yin, D., Xie, D., Sakajiri, S., Miller, C. W., Zhu, H., Popoviciu, M. L., Said, J. W., Black, K. L., and Koeffler, H. P. (2006). DLK *1*, increased expression in gliomas and associated with oncogenic activities. Oncogene *25*, 1852–1861.

Zou, H., McGarry, T. J., Bernal, T., and Kirschner, M. W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis [see comments]. Science *285*, 418–422.